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Characterization of a Chromosomal Nickel Resistance Determinant from *Klebsiella oxytoca* CCUG 15788

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***Klebsiella oxytoca* CCUG 15788 is resistant to Ni^{2+} at a concentration of 10 mM and grows in an inducible manner when exposed to lower concentrations of Ni^{2+}. The complete genomic sequence of a 4.2-kb HindIII-digested fragment of this strain was determined from genomic DNA. It was shown to contain four nickel resistance genes (*nirA*, *nirB*, *nirC*, and *nirD*) encoding transporter and transmembrane proteins for nickel resistance. When the plasmid pKOHI4, encoding *nirABCD*, was transformed into *Escherichia coli* JM109, the cells were able to grow in Tris-buffered mineral medium containing 3 mM nickel. Tn*phoA'*-1 insertion mutants in the four nickel genes *nirA*, *nirB*, *nirC*, and *nirD* showed nickel sensitivity. The *nir* genes were heterogeneously expressed in *E. coli*, suggesting functional roles of these genes in nickel resistance.**

Keywords: *Klebsiella oxytoca* CCUG 15788, KOHI4, *nir*, nickel resistance determinant, transposon mutagenesis

Plasmid-mediated resistance to nickel has been described in *Ralstonia metallidurans* CH34 and *Achromobacter xylosoxydans* 31A [10, 13]. *R. metallidurans* CH34 harbors two endogenous metal resistance megaplasmids, pMOL28 (163 kb) and pMOL30 (238 kb). One of these plasmids, pMOL28, has been reported to contain an operon responsible for inducible resistance to 3 mM nickel and 5 mM cobalt on solid and in liquid media [3, 4, 12, 13]. The *cnr* (cobalt-nickel resistance) operon on pMOL28 consists of three regulatory genes (*cnrY*, *cnrX*, and *cnrH*) and three structural genes (*cnrC*, *cnrB*, and *cnrA*). All three structural genes encode a membrane-bound three-component cation/proton antiporter, which catalyzes the energy-dependent efflux of cobalt and nickel [1]. The regulatory genes are arranged in a region upstream of the structural genes and are responsible for the inducible transcription of the structural genes [2]. *A. xylosoxydans* 31A possesses the plasmid pTOM9, which carries two loci for nickel resistance determinants, *ncc* (nickel, cobalt, and cadmium resistance) and *nre* (nickel resistance). The *ncc* operon consists of seven open reading frames (ORFs), designated *nccYXHCBAN*, and confers high-level nickel resistance. However, the *nre* genes have been reported to confer a relatively lower level of resistance to nickel [10, 11]. *Hafnia alvei* 5-5 also contains a conjugative plasmid, pEJH 501 that includes the genes *ncrA*, *ncrB*, *ncrC*, *ncrY*, and *ncrX*, which determines inducible resistance to nickel and cobalt [5, 6].

Klebsiella oxytoca CCUG 15788, isolated from a mineral oil emulsion tank in Göteborg (Sweden), was found to contain inducible nickel resistance on the chromosome [15]. The plasmid pKOHI4 carried a 4.2-kb HindIII fragment from the genomic DNA of *K. oxytoca* CCUG 15788, and when expressed in *Escherichia coli* JM109, demonstrated constitutive nickel resistance to up to 4 mM concentrations of nickel [14]. In a DNA-DNA hybridization study conducted, positive signals were shown among the 4.2-kb HindIII-digested fragment KOHI4 and two nickel resistance plasmids, pTOM9 of *A. xylosoxydans* 31A and pEJH 501 of *H. alvei* 5-5, indicating that they may have some sequence homology [6, 14].

In the present study, we report the complete nucleotide sequence of the 4.2-kb HindIII-digested fragment that was presented by Stoppel *et al.* [14]. We have further identified a chromosomal nickel resistance determinant involving four *nir* genes, based on physiological nickel minimal inhibitory concentrations (MIC) studies of wild-type and transformant cells, as well as transposon insertional mutagenesis and functional expression studies in *E. coli*.

Comparison of Growth Patterns Between Wild-Type and Transformant Cells

When *K. oxytoca* CCUG 15788 was grown in Tris-mineral medium supplemented with 0.3% (w/v) gluconate as a carbon source, and containing $NiCl_2$ at a concentration of 3 mM, the growth was delayed for 10 h without pre-

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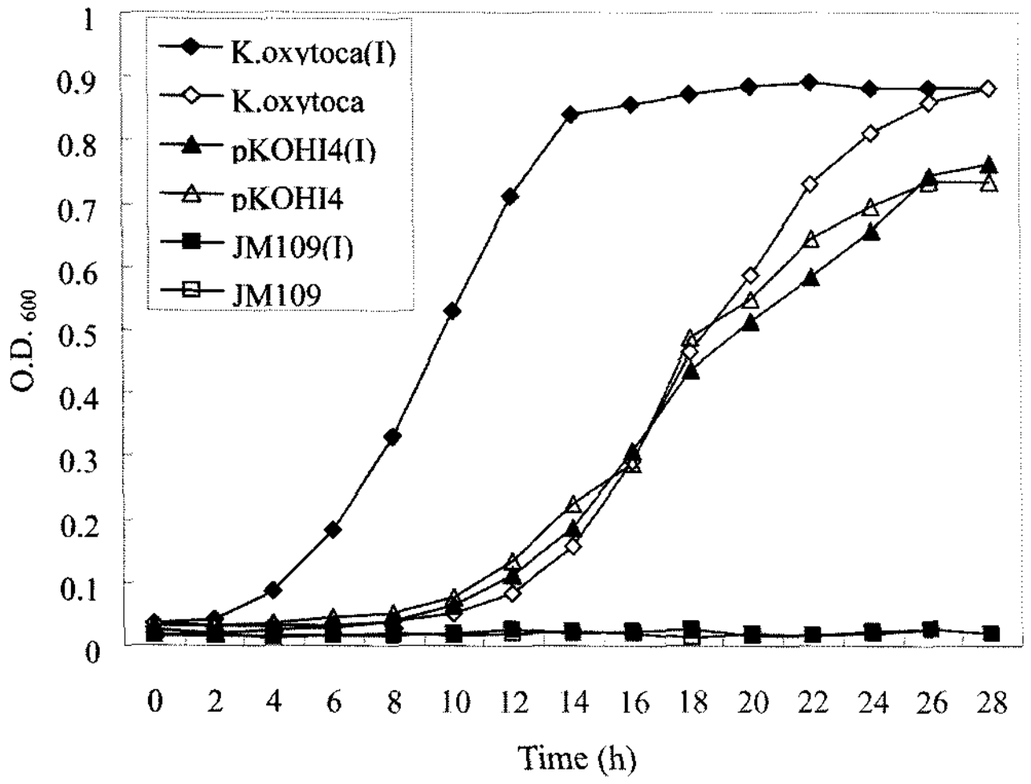

Fig. 1. Inducibility of nickel resistance of *K. oxytoca* CCUG 15788 (diamond), *E. coli* JM109 cells transformed with pKOHI4 (triangle), and *E. coli* JM109 cells transformed with the vector pBluescript SK (−) (square).
Strains were grown overnight in Tris-mineral medium [gluconate (w/v) 0.3%] without (open symbols) or with (closed symbols) 0.5 mM $NiCl_2$-induced pre-growth. Cells were then diluted 50-fold and grown in fresh medium containing 3 mM $NiCl_2$.

growth under conditions of 0.5 mM $NiCl_2$. In contrast to these results, pre-growth in media containing $NiCl_2$ at a concentration of 0.5 mM led to only two hours of lag period, and the maximal optical density was not noticeably changed (Fig. 1). *E. coli* JM109 cells containing the plasmid pKOHI4 began to grow with a 10-h delay that was also evident both in the presence and absence of pre-growth at 0.5 mM $NiCl_2$ concentrations. These results indicated that *K. oxytoca* CCUG 15788 contained a fully inducible nickel resistance determinant that responded to $NiCl_2$ as an inducer. However, *E. coli* JM109 cells harbored only structural genes for nickel resistance and these were not induced by pre-growth at 0.5 mM concentrations of $NiCl_2$.

Nucleotide Sequence and Identification of a Nickel Resistance Determinant

The complete nucleotide sequence of the 4.2-kb HindIII-digested fragment was determined. Four potential ORFs were identified by sequence analysis along with the proposed initiation codon, stop codon, ribosome-binding site, and the deduced amino acid sequence. The four ORFs were oriented in the same transcriptional direction and contained an AUG translation start codon together with a properly spaced ribosome-binding site (the nucleotide sequence of the 4,194 bp DNA fragment of the nickel resistance determinant has been deposited under the GenBank Accession No. AY492000).

The first ORF, *nirA*, encoded a protein of 356 amino acids (39 kDa, Accession No. AAR82963.1), that showed an amino acid level identity with NreB (57%) of *A. xylosoxydans* 31A and NcrA (69%)/NcrB (76%) of *H. alvei* 5-5. NreB was responsible for low-level nickel resistance by efflux and closely related to a major facilitator superfamily [15]. The second ORF, *nirB*, encoded a protein of 89 amino acids (10 kDa, Accession No. AAR82964.1) and was 74% homologous to NreA of *A. xylosoxydans* 31A in full length. The third ORF, *nirC*, corresponded to 307 amino acids (34 kDa, Accession No. AAR82965.1). The deduced amino acid sequence of NirC had a conserved region of the ABC-type transport systems such as HoxN of *R. metallidurans* and CcdA of *Bacillus subtilis* [7, 9]. The fourth ORF, *nirD*, encoded 164 amino acids (18 kDa, Accession No. AAR82966.1) and the encoded protein showed a 53% sequence homology to a possible exported protein of *Salmonella enterica*.

Recently, Tian *et al.* [17] reported a chromosomal nickel resistance determinant from *Leptospirillum ferriphilum*. This gene cluster was reported to consist of four genes, *ncrA*, *ncrB*, *ncrC*, and *ncrY*, which revealed the highest similarity in amino acids sequence identity thus far to NirA, NirB, NirC, and NirD (77.3%, 98%, 79%, and 75%) respectively.

Transposon Insertional Mutagenesis and its MIC

To map out the structural genes of the nickel resistance determinant, independent Tn*phoA'*-1 insertions in specific regions of plasmid pKOHI4 were isolated [8, 18]. The kanamycin-resistant transductants were selected after infecting cells with λP_{am}::Tn*phoA'*-1 phages at multiplicity of infection of 2.0. The resulting Tn*phoA'*-1-mutated plasmids from pools of independent kanamycin-resistants were transformed into *E. coli* JM109 cells and screened for expression of kanamycin and nickel resistance. The physical location and orientation of each insertion was mapped out using restriction enzyme digestion (Fig. 2). Nickel sensitivity with MIC values ranging from 0.25 to 1.0 mM $NiCl_2$

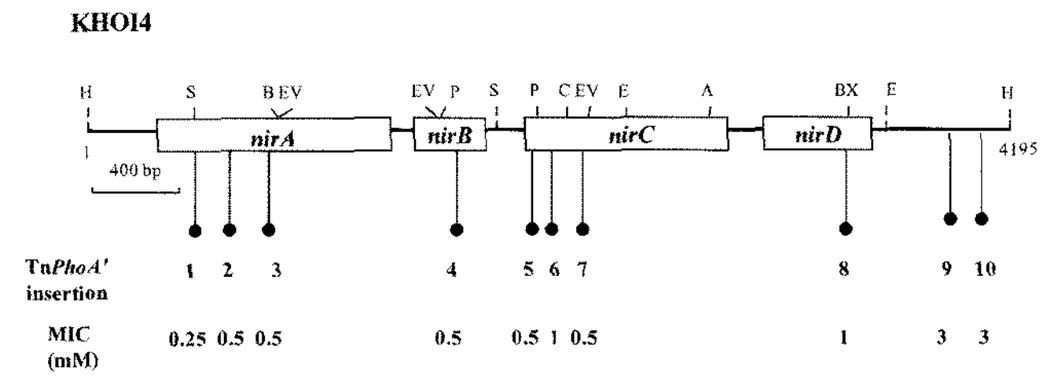

Fig. 2. Physical map of the nickel resistance determinant showing the location of Tn*phoA'*-1 insertions.
The four ORFs of the nickel resistance determinant were designated *nirA*, *nirB*, *nirC*, and *nirD*. The pinball lines represent the insertion sites of Tn*phoA'*-1 in the 4.2-kb HindIII-digested fragment of the plasmid pKOHI4. The MIC of *E. coli* JM109 cells, transformed with the pKOHI4 containing a Tn*phoA'*-1 insertion, showed nickel resistance to a range of nickel concentrations from between 0.25 to 1 mM (sites 1–8). Restriction enzyme sites: H, HindIII; S, SmaI; B, BamHI; EV, EcoRV; C, ClaI; E, EcoRI; BX, BstXI; A, ApaI; P, PvuII.

resulted from each insertion position 1 to 8 within the 4.2-kb HindIII-digested fragment. However, insertion positions 9 and 10 did not show any effect on nickel sensitivity. These results suggested that Tn*phoA'*-1 inserted the four structural genes that were essential for nickel resistance and thus showed sensitivity to nickel. Furthermore, these results did not provide any evidence for the location of a putative regulatory region between insertion sites 1 and 8.

Functional Expression of Nir Proteins

The Nir proteins were expressed in *E. coli* BL21 (DE3) cells containing the plasmid pTMJ, which contained the 4.2-kb HindIII-digested fragment that was cloned into the expression vector pT7-7, and where the *nir* genes were placed under the control of the T7 promoter [16]. Conditions of cell culture as well as induction of protein expression were followed according to a previously published method [6]. Protein samples were fractionated into supernatant and pellet fraction after centrifugation, in an attempt to roughly define the protein localization. Proteins were then analyzed on a 6–20% gradient Tris-Glycine/SDS (sodium dodecyl sulphate)-polyacrylamide gel.

Four polypeptides were functionally induced with apparent masses of about 39, 34, 18, and 10 kDa as predicted for NirA, NirC, NirD, and NirB, respectively (Fig. 3). NirA was located in both the soluble and insoluble fractions. However, NirC was exclusively located in the insoluble fraction. The hydrophobic profiles of NirA and NirC also indicated possible integral membrane proteins (data not shown). The putative NirB and NirD proteins were slightly induced in the soluble fraction. Transcription of four genes was clearly observed by RT-PCR (data not shown).

In conclusion, we have shown that the four Nir A, B, C, and D proteins were functionally expressed in *E. coli*. We further propose that NirA forms a membrane-bound complex catalyzing nickel efflux, based on the facts that NirA belongs to a major facilitator superfamily and that the C-terminal of NirA contains a histidine-rich region that might possess high affinity to nickel. NirC is a membrane protein like the ABC-type transport proteins and also possesses a histidine-rich region with high affinity for nickel binding.

The gene cluster of the chromosomal nickel resistance determinant, consisting of four structural genes, was not encoded by a regulator. The wild-type strain *K. oxytoca* CCUG 15788 was fully induced by pre-growth under conditions of 0.5 mM Ni^{2+} (Fig. 1). Thus, future studies on the regulatory mechanism of gene expression on chromosomal nickel resistance are warranted and presently under way.

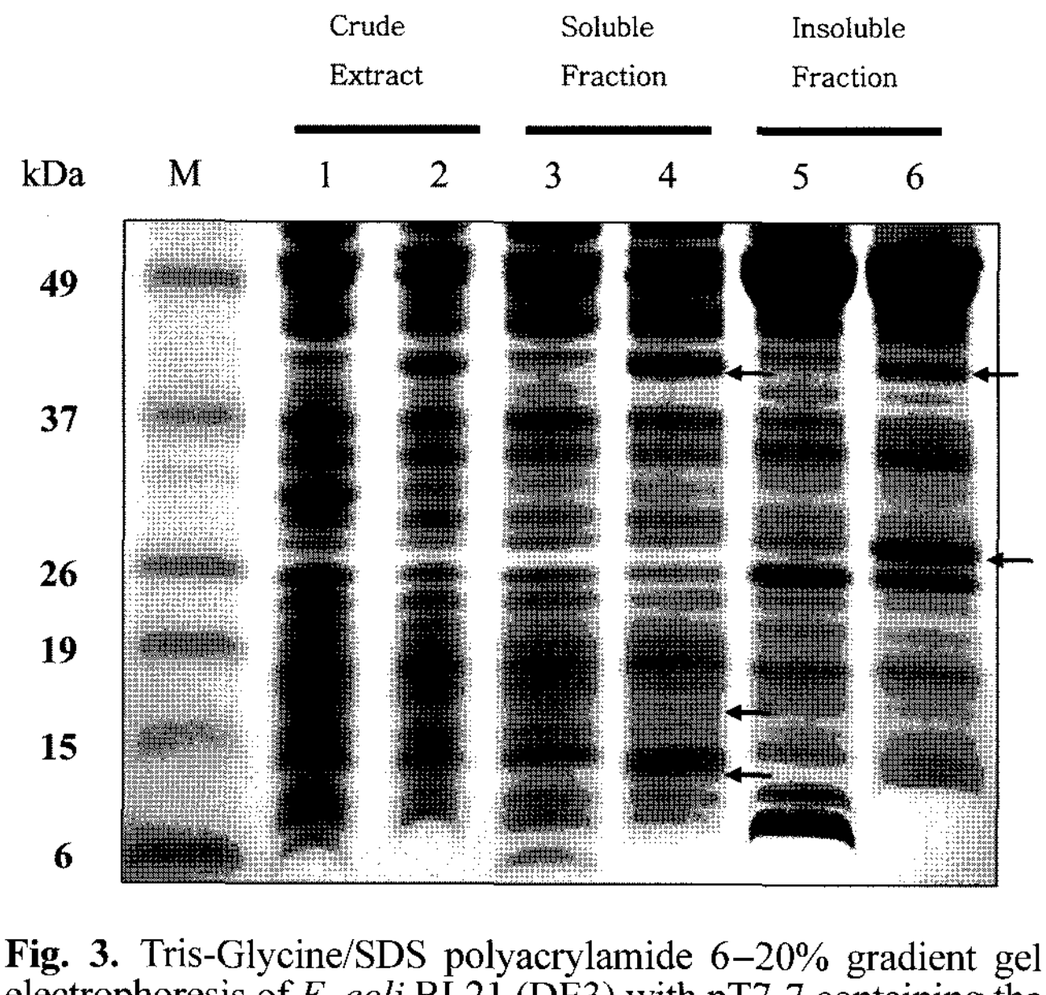

Fig. 3. Tris-Glycine/SDS polyacrylamide 6–20% gradient gel electrophoresis of *E. coli* BL21 (DE3) with pT7-7 containing the 4.2-kb full-length fragment of nickel resistance determinant (pTMJ in even numbers) and vector without insert (vector in odd numbers).
E. coli BL21 (DE3) containing pTMJ was grown in medium containing 3 mM $NiCl_2$. Protein samples were fractionated into crude extract as well as soluble and insoluble fractions after centrifugation of cells that were lysed by ultrasonication. M indicates the standard size marker.

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